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Article

Towards the anchovy biorefinery: biogas production from anchovy processing waste after fish oil extraction with biobased limonene

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Abstract: Anchovies are amid the largest fish catch worldwide. The anchovy fillet industry generates a huge amount of biowaste (e.g. fish heads, bones, tails) that can be used for the extraction of several potentially valuable bioproducts including omega-3 lipids. Following the extraction of valued fish oil rich in omega-3, vitamin D_3 and zeaxanthin from anchovy fillet leftovers using biobased limonene in a fully circular process, the solid residue was used as starting substrate for the production of biogas by anaerobic digestion. The results first reported in this study demonstrate good potential energy recovery of the anchovy sludge of about 280 $mL_{CH_4} \cdot g_{VS}^{-1}$. Due to unbalanced C/N ratio typical of marine biowaste, co-digestion with a carbon rich substrate is recommended.

Keywords: Anaerobic digestion, circular economy, biogas; fish waste, anchovy, limonene.

1. Introduction

The new and ambitious concept of the "blue economy", a paradigm founded on the biomimicry and on the sustainable exploitation of marine and natural resources calls for a collective responsibility in preserving the marine environment as one of the key factors of global prosperity [1]. In practice, action for improvement requires the design of innovative production and consumption methods with far lower environmental impact. Similar efforts are required by the 14th sustainable development goal ("Conserve and sustainably use the oceans, seas and marine resources for sustainable development") of the United Nations 2030 Agenda for Sustainable Development [2-4].

In this context, the reuse and the valorization of the fish waste is a key process not only for the reduction of its intrinsic environmental impact [5], but also for finding new economic opportunities for fisheries and fishermen. Around 35% of the global catch is either lost or wasted every year, whereas about 70% of processed fish turns into by-products (heads, viscera, skin, bones and scales) usually disposed of as waste [2]. This biowaste should rather be converted into high value bioproducts and biofuels. While the biorefinery of lignocellulosic biomasses so far, aimed to an efficient exploitation of cellulose and hemicellulose relies on well-established technologies [6-8], the complete upgrading of fish waste into biofuels, chemical intermediates, vitamins and bioactive compounds can be considered at an embryonic state, even though the number of contributions in this field of research are rapidly growing [9-11].

Certain fishery by-products are an important source of nutraceuticals and bioactive ingredients [11, 12]. Fish by-products are rich in proteins and omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs) [13].

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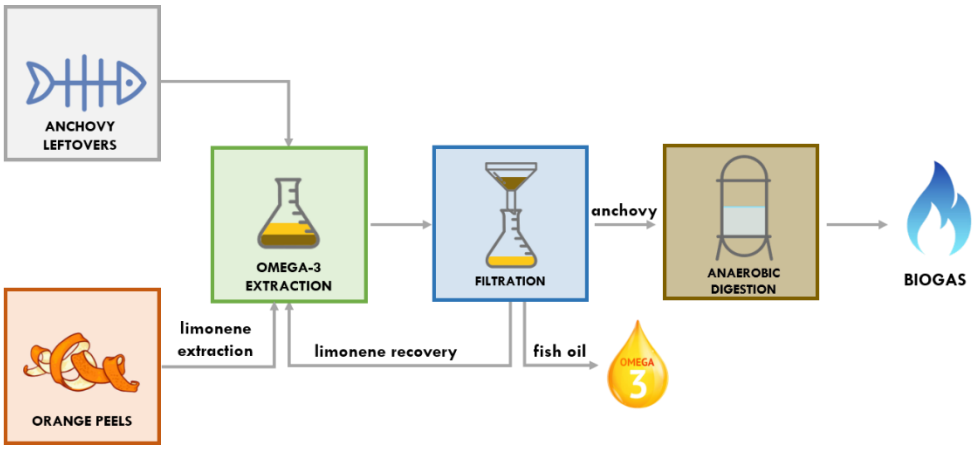
Sufficient daily intake of omega-3 marine essential lipids offers several health benefits both to adults and children and is required for the prevention of many pathologies [14, 15] As a consequence, a significant amount of the global fish catch (22 million tonnes, about 12% of the total) is used for non-food purpose, with about 1 million t of fish oil produced in the 2020.

Fish oil at industrial scale is produced with established extractive technologies including wet pressing and extraction with either organic solvent or with supercritical CO_2, followed by numerous purification steps [16]. Omega-3 concentrates, for instance, are supplied in the form of synthetic ethyl esters to which usually natural or synthetic oxidants are added to prevent quick oxidation and autooxidation of the double bonds in LC-PUFA molecular chain. Recently, a new green process for the recovery of a natural oil rich in omega-3, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), vitamin D_3 and zeaxantin has been developed by the research group of Pagliaro starting from anchovy processing waste. The method employs citrus-derived *d*-limonene as non-toxic and edible extraction solvent in a closed loop process in which the biobased solvent is fully recovered and recycled after the extraction [17,18]. The residual product derived from this new extraction process (anchovy sludge) needs to be valorized in order to close the material cycle.

Anaerobic digestion is widely recognized as one of the most effective biorefinery technologies in the upgrading of different types of organic waste and biomasses into biogas through a series of bio-chemical reactions (hydrolysis, acidogenesis and methanogenesis) occurring simultaneously in an oxygen free environment [19-21]. Together with biogas (methane: 55-70%; carbon dioxide: 30-45%; others: CO, H_2S, NH_3, H_2O, etc. [22]), a solid-liquid residue, generally known as "digestate", is produced [23]. The digestate contains macro and micro-nutrients making this by-product suitable as sustainable replacement for agricultural fertilizers [24, 25].

The sustainable production of biogas through anaerobic digestion of fish processing waste is an emerging field of research [26-29]. However, even if this biowaste is rich in lipids and proteins, the concomitant presence of LC-PUFAs, light metal ions (e.g. Ca^{2+}, Na^+, K^+, Mg^{2+}) and nitrogen-containing species (*i.e.* ammonia arisen from protein hydrolysis) can inhibit methanogenesis [30].

In this study, we present the first results on biogas production through anaerobic digestion of anchovy sludge residual from the extraction of fish oil from anchovy processing waste using limonene as solvent (Scheme 1).



Scheme 1. Biorefinery scheme for anchovy residues.

We demonstrate that methane can be obtained in good yield by using the solid residue obtained after omega-3 extraction from anchovy leftovers since the overall anaerobic digestion process is not significantly affected from the residual limonene. Moreover, the overall process is stable making the anchovy sludge an ideal substrate for co-digestion

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with other biomass wastes and residues. These results are not trivial because limonene, (and its dehydrogenation product p-cymene) is a well-known anti-microbial inhibiting the anaerobic digestion of biomass such as, for example, waste orange peel (WOP) [31,32,33]. Increasing the amount of orange essential oil (mainly composed of limonene) during the anaerobic digestion of the WOP, similar methane yields (around 370 $mL_{CH_4} \cdot g_{VS}^{-1}$) are obtained in batch tests up to highest tolerable concentration of 2000 $mg \cdot L^{-1}$ of limonene, even though at a concentration higher than 200 $mg \cdot L^{-1}$ the industrial process is, in any case, negatively affected [33].

2. Materials and Methods

All chemicals were purchased from commercial sources (Merck Life Science S.r.l. and Carlo Erba Reagents) and used without any further purification.

The fish oil extraction process was carried out following the procedure previously reported [18]. An electric blender was used to mix and homogenize the frozen anchovy leftovers (300 g) with a first aliquot of d-limonene (150 g) refrigerated at 4°C. The so-obtained semi-solid grey purées was transferred with a second aliquot of cold d-limonene (150 g) in a glass beaker sealed with an aluminum foil further coated with parafilm. The mixture was magnetically stirred at 700 rpm for 24 h at room temperature with the fish oil obtained by rotavaporing the supernatant at 90°C (pressure: 40 mbar). The solid anchovy sludge (SAS) was dried in an oven at 70°C for 3 days and, before use, was crushed in a ceramic mortar.

The morphological and elemental composition information of the solid anchovy sludge was achieved through the SEM-EDX technique with a Phenom Pro-X scanning electron microscope (SEM) equipped with an energy-dispersive X-ray (EDX) [34].

The analysis of the residual limonene present in the substrate was carried out by mixing 0.3 g of SAS with 3 mL of a solution of toluene (as internal standard) in cyclohexane (0.1 M) for six hours [35, 36]. The liquid suspension was then filtered and injected into an off-line GC-FID (Agilent 6890 N) equipped with a CP-WAX 52CB column (60 m, i.d. 0.53 mm) according to the analytical procedure already reported [37, 38].

Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) were performed on a Netzsch instrument under helium atmosphere from room temperature to 1000°C at a heating rate of 10°C/min.

The biochemical methane potential (BMP) consisting of measuring volumes of biogas and methane, produced by batches loaded with inoculum, organic substrate (SAS in this case), diluting water and nutrient solutions. The inoculum used in this experiment was a liquid digestate coming from a full-scale anaerobic digestion plant treating manure and various residues from agro-industry, located in the Reggio Calabria province (Italy). After the collection, inoculum was sieved to remove undigested materials (e. g. straw) and, then, stored at 35°C for few days until the test start. Both inoculum and substrate were characterised in terms of pH, Total Solids (TS), Volatile Solids (VS) according to standard methods [39] before the test. Moreover, only for the SAS, COD (Chemical Oxygen Demand) and C/N (Carbon/Nitrogen ratio) were measured by photometric determination (WTW Photolab S12) using specific pre-dosed cuvettes (COD Cell Test 114555) and by an elemental analyzer TOC-LCSH (Shimadzu), respectively.

The BMP test was carried out according to a method extensively used in previous studies (e. g. [39]) and in compliance with the UNI/TS 11703:2018 Italian norm and standardized protocols [41]. The method involves the use of 1.1 L glass bottles (WTW-Germany) as batches hermetically sealed. Each of them has two side necks equipped with perforable septa for biogas collection and a main central neck closed by a stopper. Bottles were placed into a thermostatic cabinet at 35 ± 0.5 °C (mesophilic conditions) and kept under continuous mixing by a magnetic stirrer. Periodically, the generated biogas was withdrawn from batches using a 100 mL syringe and transferred into an alkaline trap (NaOH solution, 3 M) where carbon dioxide was absorbed while methane caused an increase of the pressure in the trap which resulted in a displacement of an

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equal volume of solution measured in a graduated cylinder. In this way, the percentage of methane, in the generated biogas, was evaluated.

The BMP test included blank assays (in duplicates) filled with inoculum only in order to measure non-specific methane production, internal controls (in duplicates) fed with α-cellulose (CAS 9004-34-6, Sigma-Aldrich) for the validation of the process as required by the UNI/TS 11703:2018 norm, and, lastly, batches (in triplicates) loaded with SAS. Likewise inoculum and substrate, cellulose was also characterised. In each batch, volumes of inoculum, diluting water and nutrient solutions, prescribed by the aforementioned norm in order to supply macro and micro-nutrients for the bacteria metabolism, were mixed up to a working volume of 350 mL. Solutions are designed as A, B and C and contained KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, NH_4Cl (A, 5% of the total working volume), $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$ (B, 5% of the total working volume) and $MnCl_2 \cdot 4H_2O$, H_3BO_3, $ZnCl_2$, $CuCl_2$, $Na_2MoO_4 \cdot 2H_2O$, $CoCl_2 \cdot 6H2O$, $NiCl_2 \cdot 6H_2O$, Na_2SeO_3 (C, 1% of the total working volume). Amounts of cellulose and SAS (2.1 and 2.7 g, respectively) were added in order to reach a substrate to inoculum ratio (on a VS basis) equal to 0.3. Finally, the solids concentration in batches did not exceed 50 $g_{TS} \cdot L^{-1}$ as regulations recommend. Before any test start, pH of each batch mixture had been measured (Table 1).

BMP values of internal controls and substrate fed batches were expressed as the volume of produced methane gas under standard conditions (273.15 K and 101.33 kPa) per mass of VS added ($mL_{CH_4} \cdot g_{VS}^{-1}$) and determined by subtracting the average methane production of the blanks (inoculum). In accordance with regulations, the BMP test was stopped when daily methane production was lower than 1% of the total cumulated methane volume determined starting from the test beginning. This evidence emerged on day 34.

The net specific cumulative methane productions of the batches fed with SAS were modelled using the modified Gompertz equation [42]:

$$B = P \cdot exp\left\{-exp\left[\frac{R_m}{P} \cdot (\lambda - t) + 1\right]\right\}$$

where B [$L \cdot g_{VS}^{-1}$] stands for the specific methane production at time t (d); P [$L \cdot g_{VS}^{-1}$] stands for the methane production at time $t = \infty$; R_m [$L \cdot g_{VS}^{-1} \cdot d^{-1}$] stands for the maximum methane production rate; λ [d] stands for the lag phase duration. P, R_m and λ were determined the minimizing the sum of square errors between the model and the experimental average values through the Excel tool "Solver".

Table 1. Experimental reactors settings.

Substrate	Batch	Substrate [g]	TS mix	pH mix
- (blank)	1	-	2.5%	7.38
	2			7.42
Cellulose (control)	3	2.1	3.1%	7.50
	4			7.47
SAS	5	2.7	3.3%	7.39
	6			7.30
	7			7.41

At the end of any test, digestates were analysed determining pH, TS and VS [38]. Furthermore, in the liquid fraction resulting from the centrifugation (10000 rpm per 10 min) the total ammoniacal nitrogen (TAN), the Cl^- content using pre-dosed cuvettes (Ammonium Cell Test 114559 and Chloride Cell Test 114730, respectively), photometric determination (WTW Photolab S12), the total Volatile Fatty Acids (VFAs), concentration and the FOS/TAC (Volatile Organic Acids/Buffering Capacity) ratio were determined. In

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particular, the latter two parameters were determined through a four-point titration method [43] consisting of titrating 20 mL of centrifuged digestate up to pH values of 5.0, 4.4, 4.3 and 4.0 with 0.1 N sulphuric acid solution. The parameters were calculated by using the following equations [43, 44]:

$$VFAs = \left[131340 \cdot \left(V_{pH_{4.0}} - V_{pH_{5.0}}\right) \cdot \frac{N_{H_2SO_4}}{V_{sample}}\right] - \left[3.08 \cdot V_{pH_{4.3}} \cdot \frac{N_{H_2SO_4}}{V_{sample}} \cdot 1000\right] - 10.9$$

$$FOS/TAC = \frac{\left[\left(V_{pH_{4.4}} \cdot 1.66\right) - 0.15\right] \cdot 500}{V_{pH_{5.0}} \cdot 250}$$

VFAs and FOS are reported as acetic acid equivalent ($mg_{HAC} \cdot L^{-1}$) and TAC as lime equivalent ($mg_{CaCO3} \cdot L^{-1}$). $V_{pH_{5.0}}$, $V_{pH_{4.3}}$, $V_{pH_{4.4}}$ and $V_{pH_{4.0}}$ stand for the volumes recorded for acid consumptions corresponding to the respective pH values, while $N_{H_2SO_4}$ and V_{sample} represent the normality of the acid solution (0.1) and the volume in mL of the sample (20), respectively.

3. Results and discussion

SEM images clearly show the presence of an irregular and amorphous surface (Figure 2).

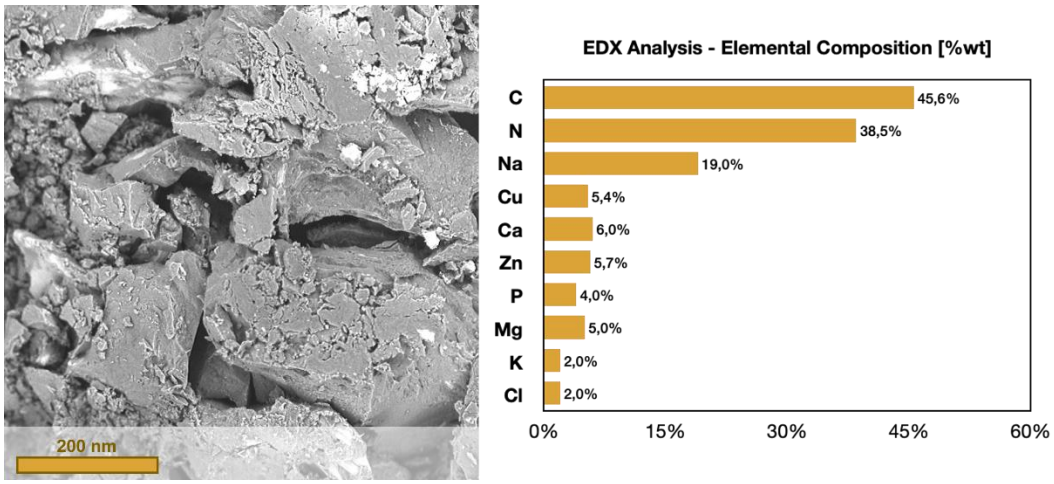

Figure 2. SEM-EDX analysis of solid anchovy's sludge.

The EDX analysis reveals the predominant presence of carbon and nitrogen. Other mineral elements including potassium, calcium, magnesium, zinc and copper are also detected. The absence of toxic and/or heavy metal contaminants (such as lead, mercury and cadmium) in the sample is worth of note [45]. This is probably due to the short life-span of small pelagic species present in the Mediterranean sea.

The thermal properties of SAS were determined by TGA and DTA analysis (Figure 3). The first degradation step (25-200°C), corresponding to a weight loss of about 10%, can be attributed to the residual water and limonene present in the sludge. The next degradation step (200-500°C), is probably due to degradation of organic materials and proteins while the last weight loss at temperature max of about 560°C is due to combustion of the remained carbon and inorganic phase (including bones and scales) [46].

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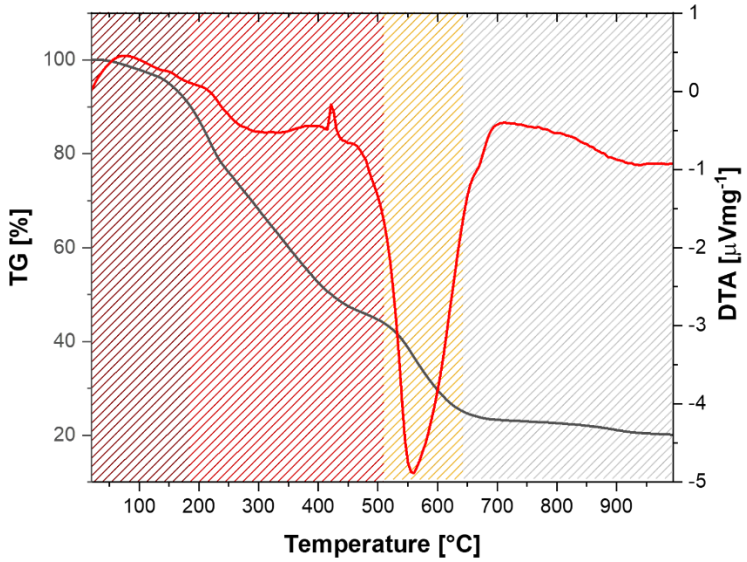

Figure 3. TGA/DTA analysis of the solid anchovy sludge.

The characterization of inoculum and substrates is summarised in Table 2.

Table 2. Characterisation of inoculum, cellulose and SAS.

	pH	TS [%]	VS [%TS]	COD [$mg_{O_2} \cdot g_{TS}^{-1}$]	C/N	d-limonene [$mg \cdot g_{TS}^{-1}$]
Inoculum	7.50	5.0 ± 0.10	76.3 ± 0.18	-	-	-
Cellulose	-	95.6	100	1185*	-	-
SAS	6.30	98.0 ± 0.15	77.1 ± 0.27	918.3	4	5

*estimated from the stoichiometry

The solids content of the SAS is quite high due to the drying carried out after the extraction process. The organic matter content, measured as VS and COD, is lower than that expected from other studies probably because of the extraction process. Furthermore, the high protein content of the fish results in a very low C/N ratio. Lastly, since the d-limonene was used as extraction solvent, its residual presence in the substrate was detected.

Table 3. Biogas and methane final productions and methane contents in biogas volumes of control and SAS fed batches.

Substrate	Batch	Biogas [$mL \cdot g_{VS}^{-1}$]	Average [$mL \cdot g_{VS}^{-1}$]	BMP [$mL_{CH_4} \cdot g_{VS}^{-1}$]	Average [$mL_{CH_4} \cdot g_{VS}^{-1}$]	Average methane content
Cellulose (control)	3	603.4	598.3	396.5	390.0	68%
	4	593.3		382.6		65%
SAS	5	406.4	378.5	296.1	278.0	72%
	6	381.7		281.3		73%
	7	347.4		256.4		73%

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Biogas productions, BMP values and average methane contents for internal control and SAS fed batches are summarised in Table 3. Average cumulated biogas and methane production trends of the three replicates fed with tested substrate are depicted in Figure 4.

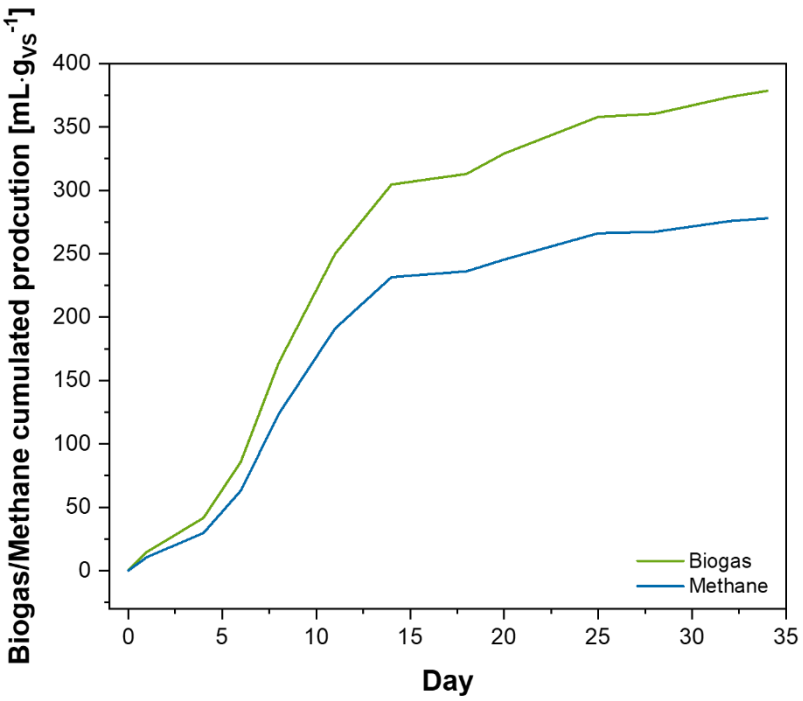

Figure 4. Cumulated biogas and methane production trends of batches fed with SAS.

The average cumulated methane production of substrate batches was modelled with the modified Gompertz equation (Figure 5) and the respective kinetic parameters calculated are summarised in Table 4.

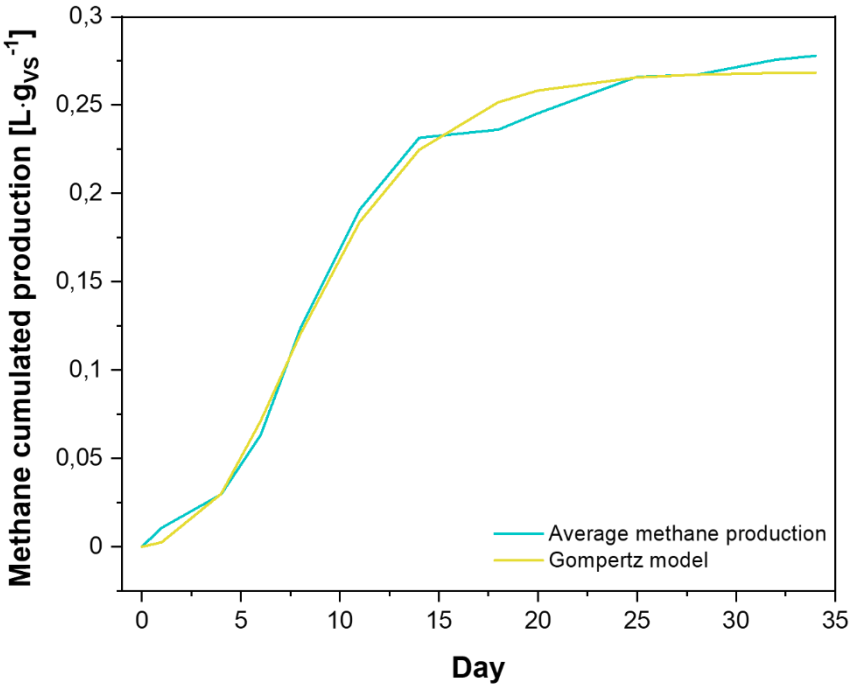

Figure 5. Simulation of cumulated methane production by Gompertz model.

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BMP values of the internal controls meet the UNI/TS 11703:2018 requirements of 325 ± 25% $mL_{CH_4} \cdot g_{VS}^{-1}$ and a difference lower than 10% was found. The results support the necessary validation of the BMP test. With regard to batches fed with SAS, batch 5 shows a slightly higher methane production than the other replicates. However, it is noticeable that final BMP values of the three batches are quite close each other indicating that the respective anaerobic processes be have uniform. Indeed, evaluating the processes in terms of biogas/methane generation, digestions performed similarly in the three replicates. In fact, after a small acclimatation period of few days, the gas production increased faster up to the 14th day. Then, batches continued to generate biogas/methane although with a slower rate until the 34th day when the test was stopped. Also, the practically identical methane contents in biogas volumes confirm that the anaerobic digestion processes of the three replicates proceeded with similar trends.

Table 4. Kinetic parameters of Gompertz equation.

P [$L \cdot g_{VS}^{-1}$]	R_m [$L \cdot g_{VS}^{-1} \cdot d^{-1}$]	λ [d]	r^2
0.269	0.025	3.145	0.997

The Gompertz interpolating model well fits the average of the experimental measurements of the three replicates ($r^2 > 0.99$). The ultimate methane production at time ∞ is predicted to be 268.7 $mL_{CH_4} \cdot g_{VS}^{-1}$ in good agreement, although slightly lower with the average BMP value at the end of the test. The lag phase duration found by using the Gompertz model of about 3 days is consistent with the initial low biogas/methane production depicted in Figure 5.

Lastly, in Table 5 chemical characteristics of the residual digestates are summarised.

Table 5. Residual digestates characterisation.

Property	Blank	Cellulose	SAS
pH	7.1 ± 0.06	7.0 ± 0.01	7.3 ± 0.00
TS	2.4 ± 0.03 %	2.5 ± 0.02 %	2.7 ± 0.01 %
VS	71.2 ± 0.19 %	72.1 ± 0.18 %	69.3 ± 0.57 %
TAN [$mg \cdot L^{-1}$]	234 ± 8.8	173 ± 8.0	697 ± 20.3
Cl^- [$mg \cdot L^{-1}$]	1263 ± 17.5	1105 ± 55.0	1297 ± 107.3
VFA [$mg_{HAC} \cdot L^{-1}$]	297.3 ± 14.11	290.4 ± 21.04	411.1 ± 28.09
FOS/TAC [$g_{HAC} \cdot g_{CaCO_3}^{-1}$]	0.08 ± 0.001	0.09 ± 0.010	0.09 ± 0.008

In contrast with blanks and controls, pH values of the mixtures of the SAS fed reactors at the end of the experiment do not vary significantly compared to those measured at the beginning of the test (Table 5). In terms of TS and VS content, no difference among the different assays were detected. Considering the initial Total Solids content of each reactor mixture, it can be noticed that in control and tested substrate fed reactors the solid matter was consumed by the microbial process while, for blanks, the solid content changed to a lower extent. The ammonium content is clearly higher in residual digestates of reactors loaded with SAS than in the others. This was predictable since the tested substrate showed a low C/N which, in the digestion process, resulted in ammonium accumulation. Differently, the determination of the Cl^- concentration does not exhibit dif-

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ferences among the different digestates. The total VFAs content was slightly higher in residual digestates of tested substrate fed reactors than in the other assays while for FOS/TAC ratios no differences among assays were observed. The very low calculated FOS/TAC ratios suggest that, for each reactor, all the organic matter was consumed by microorganisms.

SAS methane yield is consistent with the range observed for fish waste anaerobic digestion (200 – 900 $mL_{CH_4} \cdot g_{VS}^{-1}$) reported by Ivanovs et al. [47]. Comparing only studies on the anaerobic digestion of fish oil extraction residues, the BMP of our test is, by far, lower than 742 $mL_{CH_4} \cdot g_{VS}^{-1}$ (residue of salmon heads enzymatically hydrolysed in Nges et al. [28]) and 426 $mL_{CH_4} \cdot g_{VS}^{-1}$ (residue of carp viscera thermo-mechanical pre-treated in Bucker et al. [26]). In these studies, as in the present one, nitrogen inhibition was not observed despite the low C/N ratios. This was probably due to the positive inoculum influence. First of all, inoculum can contribute to balance the substrate nitrogen content as noticed by Vivekanand et al. [48] and confirmed by Bucker et al. [26]. In the aforementioned studies, methane yields of fish oil extraction residues were about 10 and 20% lower than those determined by raw fish waste digestion (828 and 541 $mL_{CH_4} \cdot g_{VS}^{-1}$ in Nges et al. [28] and Bucker et al. [26], respectively). This suggests that oil extraction does not severely affect the potential use of the fish waste as substrate in anaerobic digestion. However, further researches could be carried out in order to investigate other possible pre-treatments of both fish waste and oil extraction residues. Process conditions very similar to ours were reported by Eiroa et al. [29]. In their study on the anaerobic digestion of four different fish wastes, methane yield and final TAN content were, respectively, 285 $mL_{CH_4} \cdot g_{VS}^{-1}$ and 728 $mg \cdot L^{-1}$ (both on average). Inhibition was signalised by Morales-Polo et al. [27] where the anaerobic digestion of the anchovy waste generates only 4.6 $mL_{CH_4} \cdot g_{VS}^{-1}$ due to a distinct ammoniacal nitrogen accumulation (TAN concentration of 6.13 $g \cdot L^{-1}$).

For this reason, it is possible that co-digestion with an additional substrate having a higher C/N would be beneficial.

Considering a residual limonene content in the substrate of 5 $mg \cdot g_{TS}^{-1}$, the limonene concentration in the anaerobic mixture was around 37 $mg \cdot L^{-1}$. This value should have not negatively affected the process since, as reported in Ruiz and Flotats [31], critical concentrations are by far higher. However, the initial slow methane production and the peculiar production trend could be due to the adaptation of anaerobic microorganisms to the limonene presence as it is reported in the paper by Calabrò et al. [33].

5. Conclusions

The full valorisation of anchovy fillet processing waste requires, besides extraction of a valued fish oil rich in omega-3, vitamin D_3, and zeaxanthin, to convert the anchovy sludge residual after the lipid phase extraction. We have used the aforementioned sludge solid residue as biobased substrate in the anaerobic digestion aimed at producing biogas. A good methane yield was obtained. Residual limonene in the substrate, used as extraction solvent, did not affect the microbial methanogenesis. The good methane production potential, the process stability make this substrate ideally suited for co-digestion. Taking into account also the high nitrogen content of fish wastes, we have also demonstrated that an optimal carbon to nitrogen ratio (C/N) is advisable in order to maximize biogas production, since low C/N ratios lead to process inhibition due the pronounced toxicity of ammonia towards anaerobic digestion microorganisms responsible for methanogens.

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References

1. The Blue Economy: 10 years – 100 innovations – 100 million; https://www.theblueeconomy.org.
2. FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. https://doi.org/10.4060/ca9229en
3. United Nations. Transforming our world: the 2030 Agenda for Sustainable Development. https://www.un.org/ga/search/view_doc.asp?symbol=A/RES/70/1&Lang=E. [Accessed on 4 January 2021].
4. https://oceanconference.un.org/callforaction.
5. Calabrò, P. S.; Gori, M.; Lubello, C. (2015) European trends in greenhouse gases emissions from integrated solid waste management, Environmental Technology, 36:16, 2125-2137, DOI: 10.1080/09593330.2015.1022230
6. Xu, C.; Paone, E.; Rodríguez-Padrón, D.; Luque, R.; Mauriello, F. Reductive catalytic routes towards sustainable production of hydrogen, fuels and chemicals from biomass derived polyols. *Renewable Sustainable Energy Rev*. **2020**, *127*, 109852.
7. Xu, C.; Paone, E.; Rodríguez-Padrón, D.; Luque, R.; Mauriello, F. Recent catalytic routes for the preparation and the upgrading of biomass derived furfural and 5-hydroxymethylfurfural. *ChemSocRev* **2020**, *49*, 4273-4306.
8. Paone, E.; Tabanelli, T.; Mauriello, F. The rise of lignin biorefinery. *Curr. Opin. Green. Sustain. Chem*. **2020**, *24*, 1-6.
9. Pagliaro, M.; Pizzone, D.M.; Scurria, A.; Lino, C.; Paone, E.; Mauriello, F.; Ciriminna, R. Sustainably Sourced Olive Polyphenols and Omega-3 Marine Lipids: A Synergy Fostering Public Health. ACS Food Sci. Technol. 2021, accepted article - *in press*.
10. Maschmeyer, T.; Luque, R.; Selva, M. Upgrading of marine (fish and crustaceans) biowaste for high added-value molecules and bio(nano)-materials. Chem. Soc. Rev. 2020,49, 4527-4563.
11. Xu, C.; Nasrollahzadeh, M.; Selva, M.; Issaabadi, Z.; Luque, R. Waste-to-wealth: biowaste valorization into valuable bio(nano)materials. Chem. Soc. Rev. 2019, 48, 4791-4822.
12. Nawaz, A.; Li, E.; Irshad, S.; Xiong, Z.; Xiong, H.; Shahbazf, H.M.; Siddique, F. Valorization of fisheries by-products: Challenges and technical concerns to food industry. Trends Food Sci. Technol 2020, 99, 34-43.
13. Rizliya, V.; Mendis,E. Biological, Physical, and Chemical Properties of Fish Oil and Industrial Applications In Seafood Processing By-Products: Trends and Applications, S.-K. Kim (Ed.), Springer Science+Business Media, New York: 2014.
14. Lands, W. E. M. Fish, Omega-3 And Human Health, 2nd edition, AOCS Publishing, Urbana (IL): 2005.
15. Shahidi, F.; Ambigaipalan, P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. *Ann. Rev. Food Sci. Technol.* **2018**, *9*, 345–381.
16. Pike, I.H.; Jackson, A. Fish oil: production and use now and in the future. Lipid Technol. 2010, 22(3), 59-61.
17. Ciriminna, R.; Meneguzzo, F.; Delisi, R.; Pagliaro, M. Enhancing and improving the extraction of omega-3 from fish oil. *Sustain. Chem. Pharm.* **2017**, *5*, 54–59.
18. Ciriminna, R.; Scurria, A.; Avellone, G.; Pagliaro, M. A circular economy approach to fish oil extraction. *ChemistrySelect* **2019**, *4*, 5106 –5109.
19. Sawatdeenarunat, C.; Nguyen, D.; Surendra, K.C.; Shrestha, S.; Rajendran, K.; Oechsner, H.; Xie, L.; Khanal, S.K. Anaerobic biorefinery: Current status, challenges, and opportunities. Bioresour. Technol. 2016, 215, 304-313, doi:10.1016/j.biortech.2016.03.074.
20. Gunaseelan, V.N. Anaerobic digestion of biomass for methane production: A review. Biomass and Bioenergy 1997, 13, 83-114, doi:10.1016/S0961-9534(97)00020-2.
21. McCarty, P.L.; Smith, D.P. Anaerobic wastewater treatment. Environ. Sci. Technol. 1986, 20, 1200-1206, doi:10.1021/es00154a002.
22. Steinhauser, A.; Deublein, D. Biogas from Waste and Renewables Energy; 2011; ISBN 9783527327119.
23. Schievano, A.; Adani, F.; Tamone, F.; D'Imporzano, G.; Scaglia, B.; Genevini, P.L. What is the digestate? Anaerob. Dig. Oppor. Agric. Environ. 2009, 12.
24. Koszel, M.; Lorencowicz, E. Agricultural Use of Biogas Digestate as a Replacement Fertilizers. Agric. Agric. Sci. Procedia 2015, 7, 119-124, doi:10.1016/j.aaspro.2015.12.004.
25. Makádi, M.; Tomócsik, A.; Orosz, V. (2012). Digestate: A New Nutrient Source - Review, Biogas, Dr. Sunil Kumar (Ed.), ISBN: 978-953-51-0204-5, InTech, Available from: http://www.intechopen.com/books/biogas/digestate-a-new-nutrient-source-r. Biogas 2012.
26. Bücker, F.; Marder, M.; Peiter, M.R.; Lehn, D.N.; Esquerdo, V.M.; Antonio de Almeida Pinto, L.; Konrad, O. Fish waste: An efficient alternative to biogas and methane production in an anaerobic mono-digestion system. *Renew. Energy* **2020**, *147*, 798–805, doi:10.1016/j.renene.2019.08.140.
27. Morales-Polo, C.; Cledera-Castro, M. del M.; Hueso-Kortekaas, K.; Revuelta-Aramburu, M. Anaerobic digestion in wastewater reactors of separated organic fractions from wholesale markets waste. Compositional and batch characterization. Energy and environmental feasibility. *Sci. Total Environ.* **2020**, *726*, 138567, doi:10.1016/j.scitotenv.2020.138567.
28. Nges, I.A.; Mbatia, B.; Björnsson, L. Improved utilization of fish waste by anaerobic digestion following omega-3 fatty acids extraction. *J. Environ. Manage.* **2012**, *110*, 159–165, doi:10.1016/j.jenvman.2012.06.011.

29. Eiroa, M.; Costa, J.C.; Alves, M.M.; Kennes, C.; Veiga, M.C. Evaluation of the biomethane potential of solid fish waste. *Waste Manag.* **2012**, *32*, 1347–1352, doi:10.1016/j.wasman.2012.03.020.
30. Chen, Y.; Cheng, J.J.; Creamer, K.S. Inhibition of anaerobic digestion process: A review. Bioresour. Technol. 2008, 99, 4044–4064, doi:10.1016/j.biortech.2007.01.057.
31. Ruiz, B.; Flotats, X. Citrus essential oils and their influence on the anaerobic digestion process: An overview. Waste Manag. 2014, 34, 2063–2079, doi:10.1016/j.wasman.2014.06.026.
32. Ruiz, B.; Flotats, X. Effect of limonene on batch anaerobic digestion of citrus peel waste. Biochem. Eng. J. 2016, 109, 9–18, doi:10.1016/j.bej.2015.12.011.
33. Calabrò, P.S.; Pontoni, L.; Porqueddu, I.; Greco, R.; Pirozzi, F.; Malpei, F. Effect of the concentration of essential oil on orange peel waste biomethanization: Preliminary batch results. Waste Manag. 2016, 48, 440–447, doi:10.1016/j.wasman.2015.10.032.
34. Malara, A.; Paone, E.; Frontera, P.; Bonaccorsi, L.; Panzera, G.; Mauriello, F. Sustainable exploitation of coffee silverskin in water remediation. Sustainability 2018, 10(10), 3547.
35. Fazzino, F.; Mauriello, F.; Paone, E.; Sidari, R.; Calabrò, P.S. Integral valorisation of orange peel waste through optimized ensiling: lactic acid and bioethanol production. Chemosphere 2021, 271, 129602.
36. Calabrò, P.S.; Fazzino, F.; Folino, A.; Paone, E.; Komilis, D. Semi-continuous anaerobic digestion of orange peel waste: Effect of activated carbon addition and alkaline pretreatment on the process. Sustainability 2019, 11(12), 3386.
37. Paone, E.; Beneduci, A.; Corrente, G.A.; Malara, A.; Mauriello, F. Hydrogenolysis of aromatic ethers under lignin-first conditions. Mol. Catal. 2020, 497, 111228.
38. Gumina, B.; Espro, C.; Galvagno, S.; Pietropaolo, R.; Mauriello, F. Bioethanol Production from Unpretreated Cellulose under Neutral Selfsustainable Hydrolysis/Hydrogenolysis Conditions Promoted by the Heterogeneous Pd/Fe3O4 Catalyst. ACS omega 2019, 4(1), 352-357.
39. APHA; AWWA; WEF Standard Methods for the Examination of Water and Wastewater, 22nd Edition; E.W. Rice, R.B. Baird, A.D. Eaton, L.S.C., Ed.; American Public Health Association, American Water Works Association, Water Environment Federation, 2012; ISBN 9780875530130.
40. Calabro, P.S.; Panzera, M.F. Biomethane production tests on ensiled orange peel waste. Int. J. Heat Technol. 2017, 35, S130–S136, doi:10.18280/ijht.35Sp0118.
41. Holliger, C.; Alves, M.; Andrade, D.; Angelidaki, I.; Astals, S.; Baier, U.; Bougrier, C.; Buffière, P.; Carballa, M.; De Wilde, V.; et al. Towards a standardization of biomethane potential tests. Water Sci. Technol. 2016, 74, 2515–2522, doi:10.2166/wst.2016.336.
42. Donoso-Bravo, A.; Pérez-Elvira, S.I.; Fdz-Polanco, F. Application of simplified models for anaerobic biodegradability tests. Evaluation of pre-treatment processes. Chem. Eng. J. 2010, 160, 607–614, doi:10.1016/j.cej.2010.03.082.
43. Liebetrau, J.; Pfeiffer, D.; Thrän, D. Collection of Methods for Biogas. Collect. Methods Biogas Methods to Determ. parameters Anal. Purp. parameters that Descr. Process. biogas Sect. 2016, 1–106, doi:https://doi.org/10.1007/978-1-4939-2493-6_962-1.
44. Mézes, L.; Tamas, J.; Borbely, J. Novel approach of the basis of FOS / TAC method. Int. Symp. "Risk Factors Environ. Food Safety" "Natural Resour. Sustain. Dev. "50 Years Agric. Res. Oradea", Fac. Environ. Prot. 2011, 803–807.
45. Lozano-Bilbao, E.; Lozano, G.; Jiménez, S.; Jurado-Ruzafa, A.; Hardisson, A.; Rubio, C.; Weller, D.G.; Paz, S.; Gutiérrez, A.J. Ontogenic and seasonal variations of metal content in a small pelagic fish (Trachurus picturatus) in northwestern African waters. Mar. Pollut. Bull. 2020, 156, 111251.
46. Naga, S.M.; El-Maghraby, H.F.; Mahmoud, E.M.; Talaat, M.S.; Ibrhim, A.M. Preparation and characterization of highly porous ceramic scaffolds based on thermally treated fish bone. Ceram. Int. 2015, 41(10) Part B, 15010-15016.
47. Ivanovs, K.; Spalvins, K.; Blumberga, D. Approach for modelling anaerobic digestion processes of fish waste. Energy Procedia 2018, 147, 390–396, doi:10.1016/j.egypro.2018.07.108.
48. Vivekanand, V.; Mulat, D.G.; Eijsink, V.G.H.; Horn, S.J. Synergistic effects of anaerobic co-digestion of whey, manure and fish ensilage. Bioresour. Technol. 2018, 249, 35–41, doi:10.1016/j.biortech.2017.09.169.